

Elucidation of aspects of murine skeletal muscle regeneration using local and whole body irradiation

T. A. ROBERTSON, M. D. GROUNDS AND J. M. PAPADIMITRIOU

Department of Pathology, University of Western Australia

(Accepted 21 July 1992)

ABSTRACT

To investigate the role of proliferating local and emigrating circulatory leucocytes in skeletal muscle regeneration in mice, their bone marrow was ablated with whole body irradiation and compared with the effects of local irradiation. The results indicate that (1) the sealing of damaged myofibres is a function of local cells and is not dependent on the presence of infiltrating leucocytes; (2) the formation of sarcoplasmic projections at the ends of damaged myofibres is dependent on leucocyte infiltration; (3) nuclei in the sarcoplasmic projections are probably derived from fusion of muscle precursor cells; (4) most muscle precursor cells *in vivo* replicate at least once before fusion; and (5) both replication and fusion of muscle precursors can occur in the absence of infiltrating leucocytes. These results are discussed with respect to the interaction of various cell populations during regeneration of skeletal muscle, and are of clinical significance to pathological changes seen in many myopathies.

INTRODUCTION

Many cell types other than those derived from muscle are thought to play a role in the regeneration of skeletal muscle. Apart from nonmyogenic cells resident in skeletal muscle, those derived from the leucopoietic cell lineages including leucocytes and macrophages are implicated in skeletal muscle regeneration; however, the precise role of such cells in this process are unknown. One way of investigating the relative contribution of these cells to muscle regeneration is to prevent replication of local cells by direct irradiation, or to eliminate bone marrow derived circulating leucocytes by whole body irradiation (WBI).

There have been numerous studies on the effects of local irradiation on regenerating skeletal muscle because DNA synthesis, and hence cell replication, is impaired by high x-ray doses which rupture hydrogen bonds of the polynucleotides in double-stranded helical DNA (Andrews, 1968). The majority of investigators have demonstrated that local irradiation causes reduced phagocytic activity and a substantial decrease in numbers of muscle precursors and myotubes in the regenerating muscle (Dmitrieva, 1960; Reznik & Betz, 1967; Sloper et al. 1970; Bayliss &

Sloper, 1973; Sanes et al. 1978; Sloper & Partridge, 1980; Gulati, 1987; Wakeford et al. 1990; Weller et al. 1991). Little experimental work has been carried out, however, on the effect of WBI, except for an isolated study by Dmitrieva (1960) who examined the tibial muscles of rats after WBI (10 Gy) following trauma, and commented on the marked decrease in phagocytic activity and regeneration in these animals.

The present series of irradiation experiments was undertaken to clarify the roles of infiltrating inflammatory cells and of local cells in the early stages of muscle regeneration. In particular, we were interested in their potential effects on the sealing process of injured myofibres, and on the formation of sarcoplasmic projections extending from the ends of sealed myofibres and the origin of the myonuclei within them.

MATERIALS AND METHODS

Animals

Female inbred Swiss SJL/J mice, aged 6–8 wk and maintained as a pathogen-free colony, were used in the experiments. Mice were caged in small groups in a clean, warm animal holding facility. Each mouse was anaesthetised with halothane and the tibialis anterior

Table 1. Summary of results from irradiation experiments with muscle crush injury.

Irradiation protocol and injury time	Number of animals	Number of phagocytic cells	Number of fibroblasts	Number of myoblasts	Number of myotubes	Maximum number of nuclei in myotubes	Presence of central nuclei in myofibres	Frequency of sarco-plasmic buds/1000 fibres	Number of nuclei in sarco-plasmic buds
Unirradiated controls	4	+++	++	+++	+++	> 15	+	80	> 4
Local irradiation: injury after 24 h	4	+++	+	+	+	< 3*	+	8	1 or 2
Local irradiation 48 h after injury. Sampled at 5 d	4	+++	+	++	++	> 6	+	55	> 3
Whole body irradiation: injury within 1 h	4	+	+	±	—	N/A	+	4	1 or 2
Whole body irradiation: injury after 24 h	4	±	+	±	—	N/A	+	—	N/A
Whole body irradiation with test leg protected: injury after 24 h	4	±	++	++	++	> 10	+	—	N/A
Whole body irradiation 48 h after injury. Sampled at 5 d	4	+	+	++	+	> 5	+	40	> 4
Whole body irradiation with test leg protected. Bone marrow replenishment. Sampled at 5 d	4!	++	++	++	++	> 10	+	90	> 4
Same animals as above. Whole body irradiation with unprotected leg. Bone marrow replacement. Sampled at 5 d	4!	++	+	±	+	> 6*	+	10	1 or 2

Mice were sampled at 4 d after injury, except where otherwise indicated. Data represent pooled values for all mice.

+++ , Numerous; ++ , many; + , some; ± , occasional; — , not seen; N/A , not applicable; * , rudimentary myotube; ! , same animals used in both groups.

(TA) muscle exposed and carefully divested of its overlying fascia. After injury (see below), the wound was sutured (7.0 Ethicon). No antibiotics were administered.

Muscle injury

Crush injury. Two side-by-side crushes were made transversely across the midregion of TA using a pair of 6 inch mosquito forceps. This produced an injury approximately 4 × 5 mm wide in which overall myofibre continuity was minimally disturbed (Robertson et al. 1990).

Cut injury. A single firm incision to an approximate depth of 2 mm was made with a double-edged razor (Gillette) across the central region of the TA muscle.

Irradiation experiments

As SJL/J mice have been reported to be resistant to x-irradiation (Roderick, 1963; Yohas & Storer, 1969), a dose of 16 Gy was selected for all irradiation experiments. Mice were lightly anaesthetised with pentobarbitone sodium (Nembutal, 30 µg/g body

weight injected intraperitoneally) and exposed to the radiation source (Siemens deep x-ray; 200 kV; 1 mm Cu half value layer; 0.5 Cu filter; 8.32 Gy/min). In all experiments a continuous pliable lead sheet (2 mm thick) was used to protect parts of the body from the irradiation source. For local irradiation (to eliminate substantially replication of local resident cells within the regenerating muscle) the lead sheet was placed over the entire body with only the left leg exposed to the irradiation source. For WBI experiments (where the source of leucocytes was removed by bone marrow ablation) one leg was protected by placing the lead sheeting over the right leg only.

A complex series of experiments was carried out on 34 mice with crush injury inflicted at various times either before or after irradiation. A detailed list of the irradiation protocols used to obtain the data given in Table 1 is now presented. The numbers of animals used for each experiment are shown in Table 1.

Local irradiation

Two groups of animals were studied. (1) Leg injured 24 h after irradiation and samples taken 4 d later. (2)

Table 2. Extent of myofibre resealing using the cut injury/HRP infusion technique with a variety of irradiation protocols

Irradiation protocol	Time of injury after irradiation	Number of animals	Time of HRP infusion after injury (h)	% of resealed myofibres
Unirradiated control	N/A	2	24	95.0 ± 4.3
Local irradiation of test leg	Within 1 h	4	24	72.0 ± 9.8
Local irradiation of test leg	After 48 h	4	24	5.0 ± 4.24
Whole body irradiation with unprotected leg	Within 1 h	4	24	64.5 ± 9.1
Whole body irradiation with unprotected leg	After 48 h	4	24	6.0 ± 4.2
Whole body irradiation with unprotected leg	After 48 h	4	48	60.0 ± 8.5
Whole body irradiation with unprotected leg	After 48 h	4	72	64.5 ± 7.7
Whole body irradiation with test leg protected	After 48 h	4	24	79.5 ± 9.2

N/A, not applicable.

Leg injured, irradiated 48 h later injury and samples taken after 5 d.

Whole body irradiation

Legs unprotected. (1) Injury immediately after WBI (within 1 h) and samples taken 4 d later. (2) Injury 24 h after WBI and samples taken 4 d later. (3) Following WBI, animals were replenished with bone marrow, injured 14 d later, and samples taken after 5 d. (4) Injury followed by WBI 48 h later and sampled after 3 d.

One leg protected. (1) Injury 24 h after WBI and samples taken 4 d later. (2) Following WBI, animals were bone marrow replenished, injured 14 d later and samples taken after 5 d.

Controls. Unirradiated animals were injured and samples taken 4 d later.

Bone marrow replacement after irradiation.

Bone marrow cells were extracted from the femur of SJL/J mice as described by Grounds (1983) and 2×10^7 cells injected into each of 4 mice within 2 h of receiving WBI with one leg protected. The left and right TA of each animal was crushed 14 d later when the peripheral blood count of the animals was within normal limits.

Muscle samples from all of the crush injury experiments were processed as described below under sample preparation for electron microscopy.

Horseradish peroxidase infusion after cut injury

Experiments using diffusion of the relatively small molecular size enzyme horseradish peroxidase (HRP) into the sarcoplasm of lacerated myofibres, were performed to examine the effectiveness of sealing of

damaged myofibres at 24 h after injury. This was selected as a time when sealing should be complete, because discontinuities of the plasmalemma at the ends of damaged myofibres were not apparent by 12 h after injury (Papadimitriou et al. 1990). A precise cut injury was made on both TA muscles of 30 mice either within 1 h or at or 48 h after local or WBI (Table 2); 48 h was chosen as circulating leucocytes are effectively zero at this time. To act as controls, 2 unirradiated mice were also subjected to a cut injury. Horseradish peroxidase was infused into the cut region at 24, 48 or 72 h after injury; the numbers of animals used for each regime are summarised in Table 2. Each animal was killed 30 min after HRP injection. Based on studies of sectioned peripheral nerves (Mesulam, 1982), 30 min represents adequate time for HRP to enter unsealed damaged myofibres, and travel a significant distance into the sarcoplasm.

The TA muscles were removed, cut in half transversely through the injury site, mounted on a cork wedge so that the cut face was positioned horizontally, and the tissue frozen in isopentane which had been precooled in liquid nitrogen. Sections (5 µm) were taken deep in the frozen block, at least 300 µm beyond the area of the cut lesion, and stained using the 3–3' diaminobenzidine (DAB) technique to demonstrate HRP activity. The cut injury/infusion procedure is summarised in Fig. 1. In each specimen a substantial portion of uncut muscle was included in the block as an in-built control for endogenous peroxidase such as that associated with myoglobin. The appearance of the damaged myofibres in cross section was distinctly different to those of adjacent undamaged myofibres. In addition, muscle samples from uninjured mice and from cut injured muscles where no HRP was infused were examined for endogenous peroxidase activity.

Demonstration of HRP at the light microscope level was undertaken using a slight modification of the original method of Graham & Karnovsky (1966).

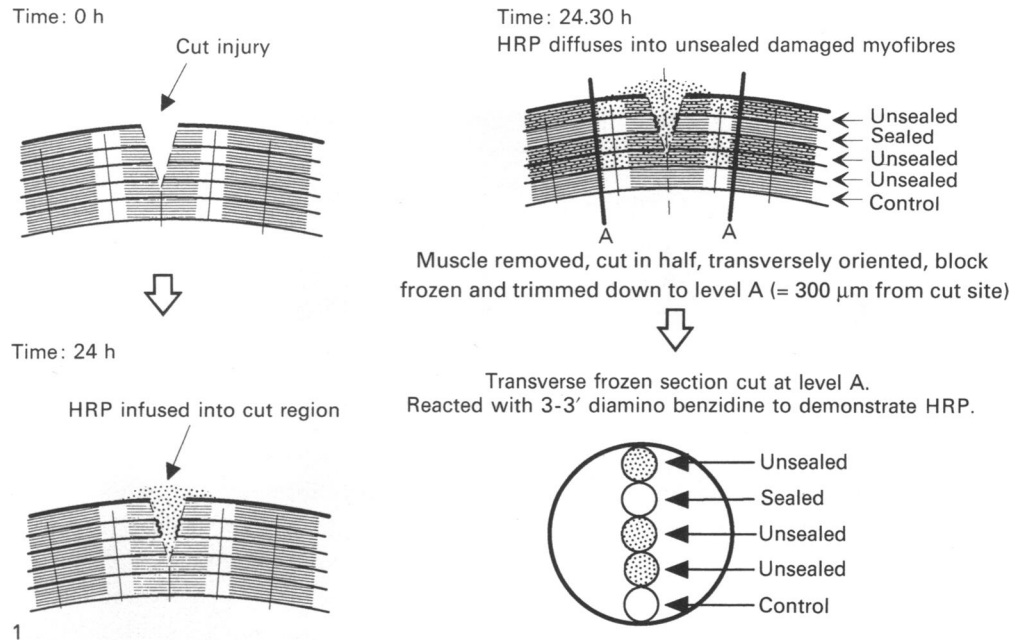


Fig. 1. Diagram showing the steps in the HRP infusion technique used to demonstrate sealed myofibres. Although the sarcomeric alignment of cut myofibres is lost by retraction, for simplicity they are depicted in alignment with neighbouring unsevered muscle. In some experiments HRP was infused at 48 h or 72 h rather than 24 h after the cut injury.

Haematological tests

White blood cell (WBC) counts and Giemsa stained peripheral blood films were examined for all animals both before and after irradiation, and at the time of killing. Blood specimens were taken from the tail vein before irradiation, and at various times after irradiation; additional samples were collected by heart puncture from animals killed at 96 h after irradiation. All specimens were collected into tubes containing the anticoagulant ethylenediamine tetraacetic acid and WBC counts were carried out on a Coulter STKS cell counter. Blood smears taken at the same time were stained with a conventional Giemsa dye for light microscopy.

Sample preparation for electron microscopy

Animals were deeply anaesthetised with halothane and perfused through the heart with 20 ml of saline containing 1000 units of heparin followed 20 ml of fixative containing 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4). Muscles were sectioned and processed for electron microscopy as described in an earlier publication (Robertson et al. 1990). Longitudinal 1 μm semithin sections were prepared from each block, stained in 0.1% toluidine blue in 5% borax, and examined by light microscopy. At least 8 blocks from each sample were sectioned. If necessary, thick slices were taken from each block with a steel

blade and additional semithin sections were prepared until suitable areas of possible interest were seen. Over 250 blocks of skeletal muscle were examined in this way to obtain the results presented in this study. In certain blocks where cell identification was difficult by light microscopy, after retrimming the block face, 50 nm ultrathin sections were cut, mounted on 200 mesh thin-bar copper grids, double stained in uranyl acetate and lead citrate, and examined in a Philips 410LS transmission electron microscope at an accelerating voltage of 80 kV.

Nomenclature

In the present study the term 'satellite cell' refers to mononuclear cells located between the sarcolemmal membrane and the external lamina of myofibres. The term sarcolemma is used in this study to describe the plasmalemmal membrane of myofibres. 'Presumptive myoblast' refers to suspected muscle precursor cell (not located in the satellite position) which lacks evidence of cytoplasmic filamentous muscle proteins; the cell contains some mitochondria, strands of endoplasmic reticulum and abundant, free ribosomes. A 'myoblast' is a muscle precursor cell confirmed by the presence of thick and thin filaments (actin and myosin) in the cytoplasm. 'Mononuclear muscle precursor cells' is a general terms which includes satellite cells, presumptive myoblasts and myoblasts. 'Myotubes' are multinucleated young muscle cells,



Fig. 2. Light micrograph of 2 cytoplasmic protrusions from a 4 d crushed nonirradiated muscle. Numerous nuclei can be seen in the stump region (arrows).

with no apparent connection to a mature myofibre. The term 'sarcoplasmic bud' was used by Hall-Craggs (1965) and 'stump' by Shafiq and Gorycki (1974) to describe a protrusion of sarcoplasm and nuclei from the injured end of a damaged myofibre (which in some instances may be very long and multinucleated). As the nature of these structures is uncertain, the term 'sarcoplasmic projection' is employed in the present study. 'Muscle cells' is an all-inclusive term which covers mononuclear muscle precursor cells, multinucleated myotubes and myofibres.

RESULTS

Muscle sampled 24 h after injury

Light or electron microscope examination of sections was not considered to be a statistically valid method of quantitating sealing of damaged myofibres. The alternative method of HRP diffusion into the sarcoplasm of sectioned fibres after cut injury was chosen to distinguish between unsealed and sealed myofibres at 24 h after injury. The results after HRP infusion into injured myofibres are summarised in Table 2. Essentially they show that substantial myofibre sealing had occurred by 24 h in animals subjected to immediate injury (within 1 h) after local or WBI. In mice subjected to local or WBI and injured 48 h later, there was by 24 h after injury almost complete failure of effective sealing. In animals which received WBI and were injured 48 h later, substantial numbers of

fibres and sealed by 48 and 72 h after injury. A significant number of sealed myofibres was also observed 24 h after injury in the protected leg of animals that had otherwise received WBI.

Muscle sampled at 4 days or more after injury

Local irradiation

When muscle from locally irradiated legs (crush injured 24 h after irradiation) was examined 4 d after injury, a prominent leucocyte infiltration was seen both in the extracellular space and within necrotic regions of damaged myofibres (after all types of injury). Ultrastructurally these infiltrating cells consisted mainly of polymorphonuclear leucocytes (PLM) and macrophages; fibroblasts and a few mast cells were also seen. Injured unirradiated control muscle had a similar appearance. The relative numbers of satellite cells and also myoblasts appeared substantially less than in nonirradiated injured controls.

Projections containing occasional nuclei were seen extending from the ends of damaged myofibres. In animals irradiated locally and with a muscle crush 24 h later, 8 projections/1000 myofibres were seen containing only 1 or 2 myonuclei. This contrasted with 80 projections/1000 myofibres containing 4-8 myonuclei observed in control crush injured animals (Fig. 2). The pooled data for each group of mice are summarised in Table 1.

In control nonirradiated injured muscle, numerous

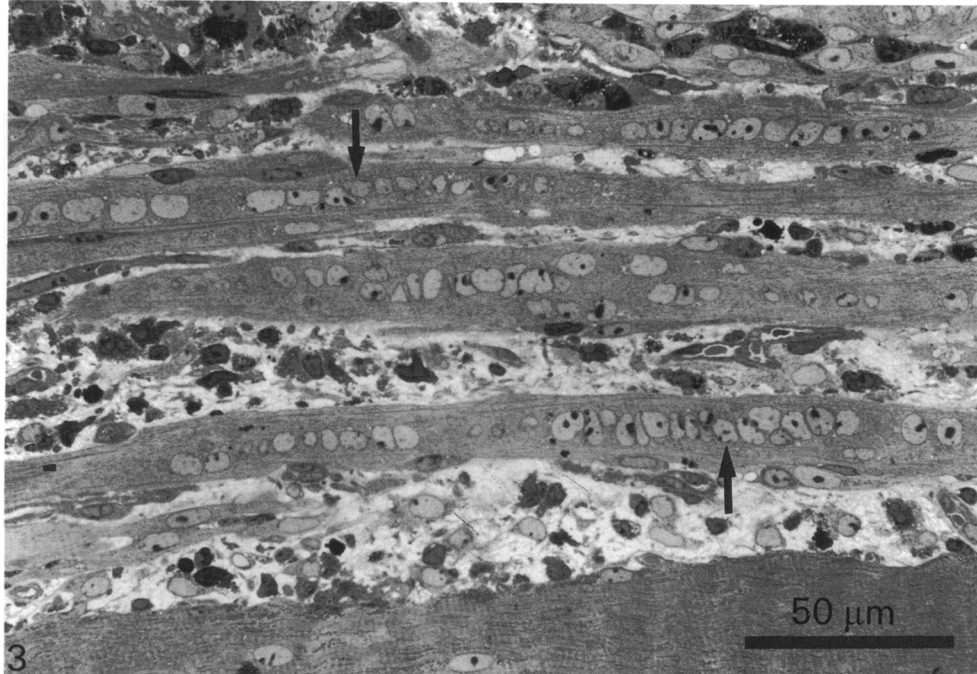


Fig. 3. Light micrograph of a group of myotubes from a 5 d crushed nonirradiated muscle. Several myotubes (arrows) contain at least 11 nuclei.

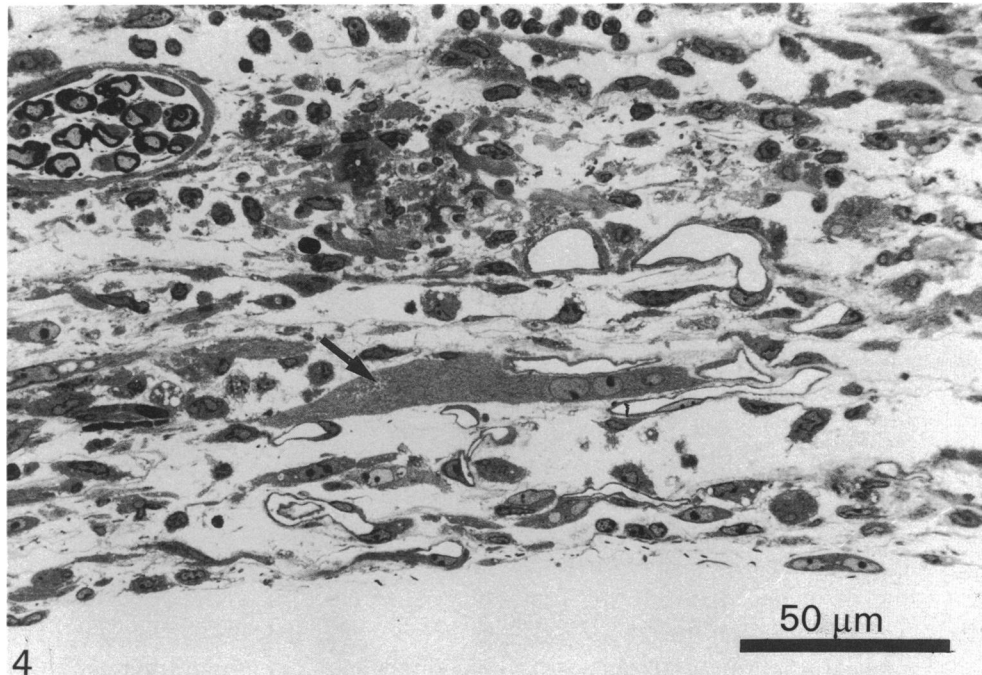


Fig. 4. Light micrograph of a rudimentary myotube (arrow) in a locally irradiated muscle 4 d after crush injury.

myotubes, each containing as many as 14 nuclei were consistently observed in samples removed at 4 or 5 d after injury (Fig. 3). In contrast only 2 rudimentary myotubes containing 2 and 3 nuclei respectively were observed in over 60 blocks prepared from locally irradiated animals (Fig. 4). Ultrastructural examination of these myotubes did not reveal any myofibrillar organisation although myofilaments were

present, and the cytoplasm contained few organelles. This contrasts with control nonirradiated injured muscles where sarcomeric differentiation and abundant cellular organelles were seen in many of the myotubes examined. Central nuclei were a consistent feature in many myofibres in both test and non-irradiated injured animals.

In animals which were locally irradiated 48 h after

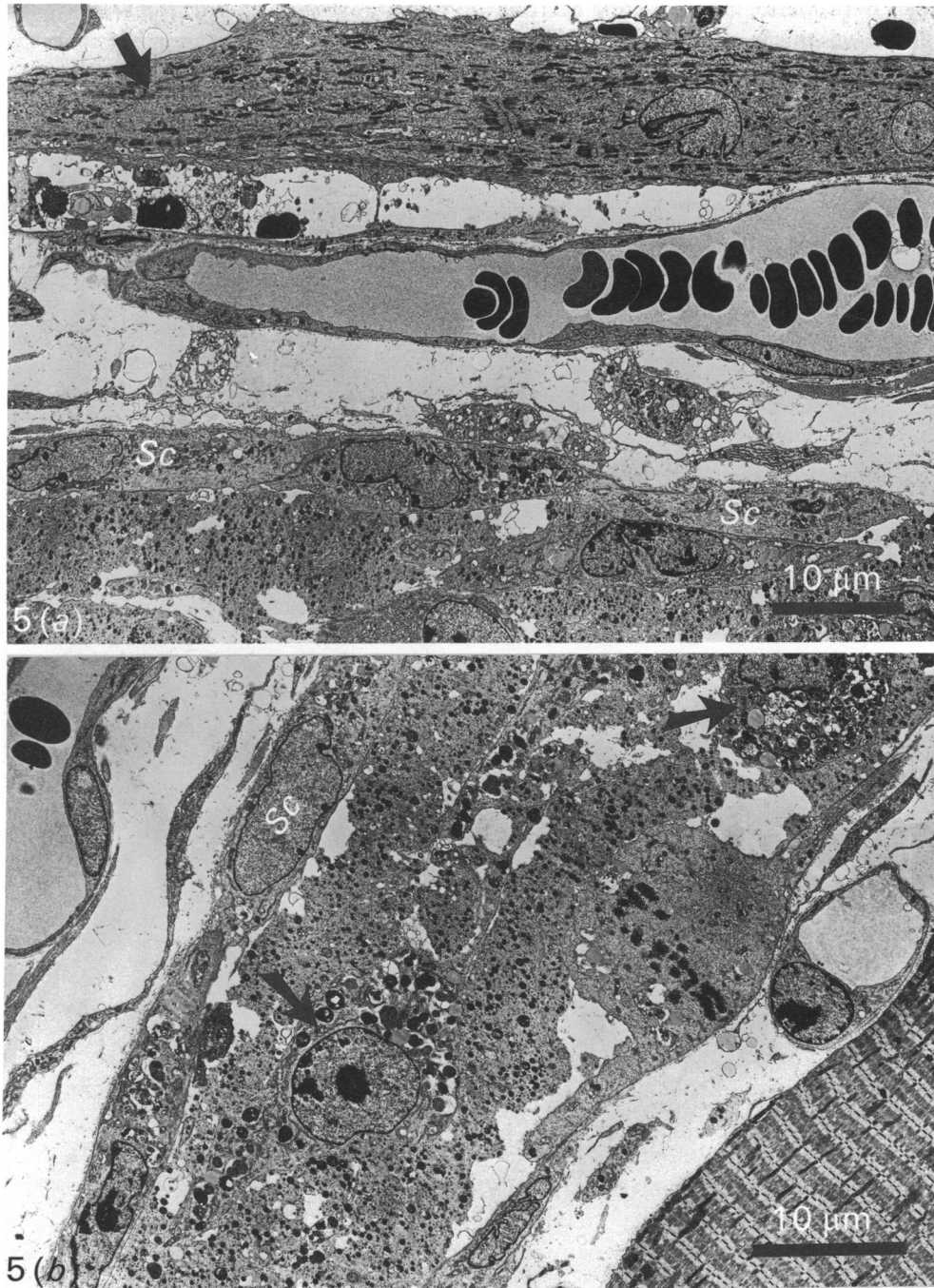


Fig. 5. Electron micrographs from the protected leg of an animal which received WBI 24 h before injury. The sample was removed 4 d after injury. Note the persisting necrotic tissue within damaged myofibres. *a*, Numerous activated satellite cells (Sc) can be observed in a necrotic myofibre and a myotube (arrow) can be seen in the extracellular space. A large blood vessel with red blood cells is also present. *b*, Activated satellite cells (Sc) and a mitotic figure can be seen beneath the external lamina of a necrotic myofibre and occasional phagocytic cells are present within the debris (arrows).

injury and killed 2 d later, phagocytosis of necrotic debris was a prominent feature in the skeletal muscle sections. Numerous macrophages were observed infiltrating damaged myofibres and many myoblasts and myotubes were also seen in these regions. The number of nuclei in myotubes was often greater than 6 and myofilaments appeared well organised. Sarcoplasmic projections were often seen containing 3 or more

myonuclei, and macrophages containing phagocytosed material were conspicuous (Table 1).

Whole body irradiation

Injury after irradiation

In muscles injured immediately after WBI and removed 4 d later, inflammatory cells including PML

and macrophages were sparse, and persisting necrotic sarcoplasmic debris was much more conspicuous than in nonirradiated muscle. When these specimens were examined by transmission electron microscopy, an occasional presumptive myoblast and fibroblast was seen in the damaged areas. The relative numbers of satellite cells in these specimens appeared lower than in the control injured animals. Only 2 sarcoplasmic projections were positively identified from 500 myofibres: they contained 1 and 2 nuclei respectively and debris was present within the sarcoplasm of each projection. Myotubes were not found. Central nuclei were apparent in many myofibres.

In animals injured 24 h after WBI and examined 4 d later, leucocyte infiltrates, sarcoplasmic projections or myotubes were never observed in the damaged areas, although an infrequent presumptive myoblast and fibroblast was present. The relative numbers of satellite cells again were much less than seen in nonirradiated injured control muscles and central nuclei were a common feature in the sarcoplasm of many intact myofibres.

In muscles injured after 24 h and removed 4 d later from the leg protected during WBI, muscle precursors and myotubes were readily found (Fig. 5*a*), although sarcoplasmic projections were never seen in any of more than 800 myofibres examined. Numerous myotubes containing more than 8 nuclei were consistently seen in damaged myofibres which still contained abundant necrotic debris, and the occasional phagocytic cell was found within the necrotic myofibres (Fig. 5*b*). Sarcomeric differentiation was apparent in many myoblasts and myotubes observed in and around the injury site. Numerous fibroblasts were seen, many of which had infiltrated the necrotic fibres. Activated satellite cells with pronounced nuclear euchromatin, prominent nucleoli, numerous mitochondria, free ribosomes and prominent strands of endoplasmic reticulum were seen in many sections. The numbers of myogenic cells including satellite cells, myoblasts and myotubes appeared less frequent than in control muscles (Table 1).

Injury before irradiation

In animals subjected to WBI 48 h after injury and sampled 3 d later (to examine the direct effect of irradiation on fusion), myoblasts and myotubes were observed, although their numbers were significantly reduced when compared with control nonirradiated muscles sampled 5 d after injury (Table 1). The number of nuclei present in these myotubes from irradiated muscles was frequently less than 4, and

Table 3. *Summary of peripheral blood counts*

Irradiation protocol	Time of sampling after irradiation (h)	WBC counts ($\times 10^3$ cells/mm ³)
Unirradiated	N/A	5.3 ± 1.72
Whole body irradiation	24	0.3 ± 0.1
Whole body irradiation	48	0
Whole body irradiation	96	0

sarcomeric filaments were sparse. Sarcoplasmic projections were commonly seen although the number of nuclei in buds was consistently less than 4. Much of the necrotic debris from damaged myofibres had been removed, although reduced numbers of phagocytic cells were seen when compared with unirradiated muscles, and many fibroblasts were observed in and around the necrotic area. In those animals locally irradiated 48 h after injury and sampled 3 d later, the cellular appearance was similar to that seen in control nonirradiated injured muscle. Although, the number of sarcoplasmic projections was marginally decreased when compared with normal injured muscle projections, were more numerous than those found in animals which were exposed to WBI 48 h after injury. The data from the above irradiation experiments examined 4 d after crush injury are summarised in Table 1.

Bone marrow replacement after irradiation

In animals in which bone marrow replenishment was carried out following WBI, and muscles injured 14 d later, 90 sarcoplasmic projections/1000 myofibres were seen (Table 1).

Haematological assessment

Results of the haematological tests on the non-irradiated and irradiated animals are presented in Table 3. They show that at 24 h after irradiation there is a marked reduction in numbers of peripheral WBC, and that peripheral WBC are not detectable by 48 h after irradiation. These results were confirmed by Giemsa staining of peripheral blood smears.

DISCUSSION

Leucocytes and fibroblasts

Local or whole body irradiation had a significant effect on numbers of leucocytic cells (including

granulocytes and monocytes) infiltrating the injured areas. As described by other investigators (Sloper & Partridge, 1980; Gulati, 1987; Wakeford et al. 1990) after local irradiation there was a decrease of both PML and macrophages in the necrotic area. After WBI, phagocytic cells were present in necrotic areas when crush injury was inflicted immediately after irradiation (similar to the numbers seen in non-irradiated, injured control animals); however, there was an almost total absence of these cells where injury to the muscle was delayed until 24 h or more after WBI. These results indicate that PML and monocyte migration from intact blood vessels was not impaired at the initial time of WBI, even though macrophages were no longer capable of replication.

The reduction in the WBC count from around 5000/mm³ in unirradiated mice to 300/mm³ at 24 h after WBI, and the absence of WBC in peripheral blood by 48 h after WBI, is in agreement with the susceptibility of leucocytes to irradiation. In non-irradiated mice, lymphocytes represent approximately 79%, polymorphonuclear leucocytes (PML) 19%, and monocytes 2% of the leucocyte population (Schalm et al. 1975). The lymphocyte is extremely sensitive to ionising radiation and loss of the normal nuclear pattern can be observed by light microscopy within an hour of irradiation. Complete dissolution follows, with rapid disappearance from the peripheral blood (Loutit, 1962). The half-life of PML has been estimated as 6 h (Schalm et al. 1975) with a cell turnover of 80% every 48 h as demonstrated by tritiated thymidine experiments on vascular leucocytes (Bintliff & Walker, 1960). Although the half-life of peripheral blood monocytes is approximately 3 d (Whitelaw, 1966), the half-life of tissue macrophages is much longer and has been estimated at between 10 d to 1 y. After WBI the rapid disappearance of WBC from the peripheral blood (within 24 h) is accounted for by leakage of such cells from the gut and other organs without replenishment from bone marrow stem cells. This would explain the lack of leucocytes in the crush lesions inflicted 24 h after irradiation. Macrophages are thought to play an important role in muscle repair as, apart from their phagocytic potential, they secrete a wide range of enzymes and growth factors associated with efficient muscle regeneration (Nathan, 1987; Papadimitriou & Ashman, 1989; Grounds, 1991).

It was particularly interesting that in mice that received WBI with one leg protected and a muscle crush in the protected leg 24 h later (at a time when there were very few circulating leucocytes), many myoblasts and myotubes were present in the lesion

examined 4 d later. This important observation shows that proliferation and fusion of muscle precursor cells is not dependent on the presence of infiltrating macrophages or other leucocytes. In an autoradiographic study of whole muscle grafts it was shown that the replication of muscle precursor cells was closely associated with, but preceded, the influx of macrophages and revascularisation (Roberts & McGeachie, 1990).

This result, in conjunction with the finding that the onset of replication is earlier in crush injured muscles (where vascularisation is relatively intact) compared with grafted muscles where revascularisation must occur (Grounds & McGeachie, 1990), indicates that diffusible substances derived either from infiltrating leucocytes or from ingrowing blood vessels are required for the synthesis of DNA by muscle precursor cells after trauma. The results of the present study (where replication occurred in the absence of infiltrating leucocytes) favours a role for soluble factors, derived either from the circulation or from local cells, in the onset of muscle precursor replication. This is in agreement with a study by Bischoff (1990a) who demonstrated that, although a factor derived from injured muscle is capable of triggering satellite cell activation, a further serum-derived factor is essential for maintaining progression through the cell cycle and satellite cell proliferation. In addition, in the WBI experiment where one leg was protected, the relative absence of macrophages and PML but the presence of fibroblasts during the time (48 h and onwards) of myoblast differentiation and fusion and myotube maturation, suggests the possibility that fibroblasts may participate in these aspects of myogenesis. Fibroblasts secrete many growth factors and extracellular matrix components known to affect myogenic cells (Krieg & Heckmann, 1989; Quin et al. 1990; Florini et al. 1991; Grounds, 1991).

Sealing of damaged myofibres

To our knowledge there have been only 2 ultrastructural studies in the literature on the sealing of myofibres *in vivo* following injury. One was a study by Carpenter & Karpati (1989) using a microneedle injury and the second was an investigation by our group (Papadimitriou et al. 1990) in which we showed that formation of demarcating (sealing) membranes in injured muscle fibres is closely associated with the presence of activated Golgi, phospholipid vesicles and mitochondria at the injury site. These morphological features were seen in samples from all irradiation protocols, both local and WBI (data not shown),

indicating that generally these cellular events are unaffected by irradiation, and that alone they are insufficient to result in effective sealing within 24 h. The greatly reduced numbers of sealed fibres after local irradiation can probably be accounted for by impaired function of the injured myofibres, as high dose irradiation affects normal protein synthesis of cells.

The observation (from the HRP experiments) that myofibres were efficiently sealed in muscles injured within 1 h of WBI, but remained unsealed when muscles were injured 48 h after WBI (when circulating leucocyte numbers are essentially zero) initially suggested a role for blood-borne inflammatory cells. It is possible that a factor secreted from an infiltrating cell such as a PML, monocyte or lymphocyte might contribute to the sealing process; however, this cannot account for the successful sealing seen in protected legs injured at 48 h after WBI, nor the lack of sealing in muscles injured 48 h after local irradiation. An alternative explanation which we favour and which accounts for the local and WBI results is that new plasmalemmal formation is purely a function of local cells, and that this is progressively impaired at increasing times after irradiation. In animals that received WBI and were injured 24 h later, sealing was almost totally absent at 24 h after injury, although significant sealing of damaged myofibres had occurred by 48 to 72 h after injury. This shows that the sealing process is slowed but not prevented by irradiation, indicating a degree of myofibre dysfunction because of irradiation.

Formation of sarcoplasmic projections

Sarcoplasmic projections that contain a variable number of nuclei are often seen at the ends of sealed myofibres (Shafiq, 1970). It was the regular observation of these structures in regenerating muscle in the past that led to a major controversy. Many research workers supported a *continuum* theory of muscle regeneration in which it was proposed that muscle regenerated by an outgrowth of sarcoplasm with migration of myonuclei into these areas (reviewed by Stockdale & Holtzer, 1961) and there is still support for aspects of this model (Bischoff, 1990*b*; Hinterberger & Barald, 1990). The alternative *discontinuous* theory states that mononuclear muscle precursor cells fuse together to form myotubes and with the ends of myofibres. The latter theory is widely supported and accepted by the majority of investigators (reviewed by Allbrook, 1981; Roth & Oron, 1985; Schmalbruch, 1986). These sarcoplasmic stumps

were consistently seen in our semithin sections of normal regenerating muscle and we wished to know whether they could be formed without division of muscle precursor cells. Animals were irradiated to prevent cell replication and then muscles subjected to a crush injury, using various protocols. The reduced number of sarcoplasmic projections with diminished numbers of nuclei in stumps after local irradiation, and in muscles injured immediately after whole body irradiation, strongly supports the idea that these nuclei are derived from a proliferating population of local muscle precursors which fuse the ends of damaged myofibres. Alternatively, the muscle fibres might be damaged by irradiation such that myonuclei could not be propelled to the final site. Our results cannot distinguish between the possibility that the nuclei in sarcoplasmic projections of these irradiated animals are derived from nuclei of muscle precursors which fused without first undergoing a cell division, or from (postmitotic) migrating myonuclei. A report by Roth & Oron (1985) where sarcoplasmic projections could not be demonstrated when vinblastine (a known inhibitor of cell division) was injected before and up to 4 d after a cut injury, also supports the proposal that cell proliferation is an essential step in the formation of these myofibre extensions.

The absence of sarcoplasmic projections in injured muscles of legs protected at the time of WBI suggested that leucocytes might act as chemoattractants for directional sarcoplasmic extrusion and the formation of projections. This proposed role of leucocytes is supported by the higher number of projections in muscles subjected to local irradiation 48 h after crush injury, compared with the number in animals that received WBI at a similar time.

Strong support for a role of infiltrating leucocytes in the formation of sarcoplasmic projections comes from experiments where the bone marrow of irradiated mice was replaced. Large numbers of projections (similar to those in control muscles) were present in the protected leg of bone marrow replenished animals and some were also seen in the unprotected legs of the same animals. Furthermore these experiments also indicate that irradiation itself does not impair the formation of projections.

The numbers of muscle precursors available for fusion should theoretically be very similar in animals injured 24 h after (local) irradiation and those injured within 1 h after (whole body) irradiation, since previous autoradiographic studies show that precursor replication in SLJ/J mice does not occur before 24 h after injury (Grounds & McGeachie, 1989*a*). Although the number of projections and the number

of nuclei in projections was very low in the WBI mice compared with locally irradiated muscle or WBI mice where bone marrow was replaced, it was not statistically possible to quantitate this due to the small numbers involved.

The reasons for some centrally located nuclei seen in myofibres in both irradiated and nonirradiated animals as early as 24 h after injury are unknown. This clearly is not the result of myofibre regeneration, since this is impaired in irradiated muscle and new myofibre formation with associated central nuclei does not occur by this time in normal muscle. Thus movement of the myonuclei from their peripheral position appears to be a rapid response of the myofibre to damage.

Myotube formation

In animals in which skeletal muscle was subjected to local irradiation 24 h or immediately before injury, the presence of occasional rudimentary myotubes confirms that some, but very few, muscle precursor cells can fuse without cell replication. This is of interest with respect to autoradiographic studies which determined the number of cell divisions before fusion of muscle precursor cells in regenerating muscle. Since these studies required the uptake of tritiated thymidine by replicating cells it was not possible to identify, or comment upon, how many muscle precursors fused without first replicating (Grounds & McGeachie, 1989*b*). To test the possibility that fusion of muscle precursor cells (which had not undergone mitosis) might have been impaired by irradiation, regenerating muscle was irradiated at a time when muscle precursor cells have normally replicated and are starting to fuse (48 h after injury). The presence of many myotubes in such irradiated muscles sampled 5 d after injury, excludes the possibility that irradiation prevents myogenic fusion and lends strong support to the idea that most muscle precursor cells replicate at least once before fusion *in vivo*.

Conclusions

These studies using various protocols of local and WBI have clarified the role of local cells and infiltrating leucocytes in various aspects of skeletal muscle regeneration *in vivo*. From our data we hypothesise that myofibre sealing is dependent upon myofibre integrity only, myogenic cell proliferation and fusion is not dependent on circulating leucocytes although a serum or local factor cannot be excluded,

and sarcoplasmic projection formation depends on both the presence of leucocytes and fusion of muscle precursor cells.

ACKNOWLEDGEMENT

Support from the National Health and Medical Research Council of Australia (M. D. G.) is gratefully acknowledged.

REFERENCES

- ALLBROOK D (1981) Skeletal muscle regeneration. *Muscle & Nerve* **4**, 234–245.
- ANDREWS, JA (1968) Cell killing by ionizing radiations. In *The Radiobiology of Human Cancer Radiotherapy* (ed. J. A. Andrews). Philadelphia: W. B. Saunders.
- BAYLISS LM, SLOPER JC (1973) The role of the circulating cell as a source of myoblasts in the repair of injured skeletal muscle: evidence derived from the preirradiation of injured tissue and the use tritiated thymidine. In *Basic Research in Myology* (ed. B. A. Kakulas), pp. 346–349. Amsterdam: Excerpta Medica.
- BINTLIFF S, WALKER BE (1960) Radioautographic study of skeletal muscle regeneration. *American Journal of Anatomy* **106**, 233–245.
- BISCHOFF R (1990*a*) Cell cycle commitment of rat muscle satellite cells. *Journal of Cell Biology* **111**, 201–207.
- BISCHOFF R (1990*b*) Interaction between satellite cells and skeletal muscle. *Development* **109**, 943–952.
- CARPENTER S, KARPATI G (1989) Segmental necrosis and its demarcation in experimental micropuncture. *Journal of Neuro-pathology and Experimental Neurology* **48**, 154–170.
- DMITRIEVA EV (1960) Post-traumatic regeneration of skeletal type musculature under total and local X-irradiation. *Arkhiv Anatomic Gistologii Embriologii* **39**, 11–22 (in Russian, English summary).
- ECHEVERRIA OM, VAZQUEZ-NIN GH, NINOMIYA JG (1983) Structural alterations and changes in the distribution of markers in transected skeletal muscle fibers in the guinea pig. *Acta Anatomica* **116**, 358–365.
- ECHEVERRIA OM, NINOMIYA JG, VAZQUEZ-NIN GH (1987) Microscopical and electrophysiological studies on the healing-over of striated fibers of cremaster muscle of the guinea pig. *Acta Anatomica* **128**, 278–280.
- FLORINI JR, EWTON DZ, MAGRI KA (1991) Hormones, growth factors, and myogenic differentiation. *Annual Reviews of Physiology* **53**, 201–216.
- GRAHAM RC, KARNOVSKY MJ (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *Journal of Histochemistry and Cytochemistry* **14**, 291–302.
- GROUNDNS MD (1983) Skeletal muscle precursors do not arise from bone marrow cells. *Cell and Tissue Research* **234**, 713–722.
- GROUNDNS MD (1991) Towards understanding skeletal muscle regeneration. *Pathological Research Practice* **187**, 1–22.
- GROUNDNS MD, MCGEACHIE JK (1989*a*) Myogenic cells in regenerating adult chicken muscle can fuse into myotubes after single cell division *in vivo*. *Experimental Cell Research* **180**, 429–439.
- GROUNDNS MD, MCGEACHIE JK (1989*b*) A comparison of muscle precursor replication in crush-injured skeletal muscle of Swiss and BalbC mice. *Cell and Tissue Research* **255**, 385–391.
- GROUNDNS MD, MCGEACHIE JK (1990) Myogenic cell replication in minced skeletal muscle isografts of Swiss and BalbC mice. *Muscle & Nerve* **13**, 305–313.
- GULATI AK (1987) The effect of X-irradiation on skeletal muscle regeneration in the adult rat. *Journal of the Neurological Sciences* **78**, 111–120.

- HALL-CRAGGS ECB (1965) The regeneration of skeletal muscle fibres per continuum. *Journal of Anatomy* **117**, 171–178.
- HINTERBERGER TJ, BARALD KF (1990) Fusion between myoblasts and adult muscle fibres promotes remodeling of fibres into myotubes in vitro. *Development* **109**, 139–148.
- KRIEG T, HECKMANN M (1989) Regulatory mechanisms of fibroblast activity. *Recent Progress in Medicine* **80**, 594–598.
- LOUTIT JF (1962) In *Irradiation of Mice and Men*, p. 6. Chicago: University of Chicago Press.
- MESULAM M (1982) Principles of horseradish peroxidase neuro-histochemistry and their applications for tracing neural pathways, axonal transport, enzyme histochemistry and light microscopic analysis. In *Tracing Neural Connections* (ed. M. Mesulam), pp. 3–152. New York: Wiley.
- NATHAN CF (1987) Secretory products of macrophages. *Journal of Clinical Investigation* **79**, 319–326.
- PAPADIMITRIOU JM, ASHMAN RB (1989) Macrophages: current views on their differentiation, structure and function. *Journal of Ultrastructural Pathology* **13**, 343–372.
- PAPADIMITRIOU JM, ROBERTSON TA, MITCHELL CA, GROUNDS MD (1990) The process of new plasmalemmal formation in focally injured skeletal muscle. *Journal of Structural Biology* **103**, 124–134.
- QUIN LS, ONG LD, ROEDER RA (1990) Paracrine control of myoblast proliferation and differentiation by fibroblasts. *Developmental Biology* **140**, 8–19.
- REZNIK M, BETZ EH (1967) Influence de l'irradiation locale préalable sur les capacités régénératrices du muscle strié squelettique. *Pathologie Européenne* **2**, 69–80.
- ROBERTS P, MCGEACHIE JK (1990) Endothelial cell activation during angiogenesis in freely transplanted skeletal muscles in mice and its relationship to the onset of myogenesis. *Journal of Anatomy* **169**, 197–207.
- ROBERTSON TA, GROUNDS MD, MITCHELL CA, PAPADIMITRIOU JM (1990) Myogenic fusion in vivo. *Journal of Structural Biology* **105**, 170–182.
- RODERICK TH (1963) The response of 27 inbred strains of mice to daily doses of whole body x-irradiation. *Radiation Research* **20**, 631–639.
- ROTH D, ORON U (1985) Repair mechanisms involved in muscle regeneration following partial excision of the rat gastrocnemius muscle. *Experimental Cell Biology* **53**, 107–114.
- SANES J, MARSHALL LM, MACMAHON VJ (1978) Reinnervation of muscle fibre basal lamina after removal of myofibres. *Journal of Cell Biology* **78**, 176–198.
- SCHALM OW, JAIN NC, CARROLL EJ (Eds) (1975) In *Veterinary Haematology*. Philadelphia: Lea and Febiger.
- SCHMALBRUCH H. (1986) Muscle regeneration: fetal myogenesis in a new setting. *Bibliotheca Anatomica* **29**, 126–153.
- SHAFIQ SA (1970) Satellite cells and fibre nuclei in muscle regeneration. In *Regeneration of Striated Muscle* (ed. A. Mauro, S. A. Shafiq & A. T. Milhorat) pp. 122–132. Amsterdam: Excerpta Medica.
- SHAFIQ SA, GORYCKI MA (1965) Regeneration in skeletal muscle of mouse: some electron-microscopical observations. *Journal of Pathology and Bacteriology* **90**, 123–127.
- SLOPER JC, BATESON R, HINDLE D, WARREN J (1970) Muscle regeneration in man and the mouse: evidence derived from tissue culture, and from the evolution of experimental and surgical injuries in the irradiated and non-irradiated subject. In *Skeletal Muscle and Myogenesis*. (ed. A. Mauro, S. A. Shafiq & A. T. Milhorat), pp. 157–164. Amsterdam: Excerpta Medica.
- SLOPER JC, PARTRIDGE TA (1980) Skeletal muscle: regeneration and transplantation. *British Medical Bulletin* **36**, 153–158.
- STOCKDALE FE, HOLTZER H. (1961) DNA synthesis and myogenesis. *Experimental Cell Research* **24**, 508–520.
- WAKEFORD S, WATT DH, PARTRIDGE TA (1991) X-irradiation improves mdx mouse muscle as a model of myofibre loss in DMD. *Muscle and Nerve* **14**, 42–50.
- WELLER B, KARPATI G, LEHNERT S, CARPENTER S. (1991) Major alteration of the pathological phenotype in gamma irradiated soleus muscles. *Journal of Neuropathology and Experimental Neurology* **50**, 419–431.
- WHITELAW DM (1966) The intravascular lifespan of monocytes. *Blood* **28**, 455–464.
- YOHAS JM, STORER JB (1969) On mouse strain differences in radiation resistance: hematopoietic death and the endogenous colony forming unit. *Radiation Research* **39**, 608–622.